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CTG repeat-targeting oligonucleotides for down-regulating Huntingtin expression

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ABSTRACT

Huntington's disease (HD) is a fatal, neurodegenerative disorder in which patients suffer from mobility, psychological and cognitive impairments. Existing therapeutics are only symptomatic and do not significantly alter the disease progression or increase life expectancy. HD is caused by expansion of the CAG trinucleotide repeat region in exon 1 of the Huntingtin gene (*HTT*), leading to the formation of mutant *HTT* transcripts (mu*HTT*). The toxic gain-of-function of muHTT protein is a major cause of the disease. In addition, it has been suggested that the mu*HTT* transcript contributes to the toxicity. Thus, reduction of both muHTT mRNA and protein levels would ideally be the most useful therapeutic option. We herein present a novel strategy for HD treatment using oligonucleotides (ONs) directly targeting the *HTT* trinucleotide repeat DNA. A partial, but significant and potentially long-term, HTT knock-down of both mRNA and protein was successfully achieved. Diminished phosphorylation of *HTT* gene-associated RNA-polymerase II is demonstrated, suggestive of reduced transcription downstream the ON-targeted repeat. Different backbone chemistries were found to have a strong impact on the ON efficiency. We also successfully use different delivery vehicles as well as naked uptake of the ONs, demonstrating versatility and possibly providing insights for *in vivo* applications.

INTRODUCTION

Huntington's disease (HD) is a fatal, neurodegenerative disorder affecting the striatum, cerebral cortex and other subcortical structures. HD onset appears around midlife in most cases, and is characterized by a combination of symptoms: movement abnormalities, emotional disturbances and cognitive impairments (1). The most characteristic feature in HD patients is the uncoordinated irregular movements, known as 'chorea' (Greek for: dance); however, this is usually preceded by psychiatric symptoms and cognitive problems. In most cases, HD symptoms begin at 35–50 years of age and end with death 15–20 years later (2). Despite the identification of the genetic cause of HD in 1993 (3), no curative therapy has yet been developed. Current pharmaceuticals can only provide symptomatic amelioration but fail to treat the underlying cause or stop disease progression (4).

HD is caused by a polyglutamine-encoding CAG trinucleotide repeat expansion in exon 1 of the Huntingtin (*HTT*) gene leading to the formation of mutant HTT (muHTT) protein. The number of the repeats in the mutant allele inversely correlates with the age of onset. The prevalence of HD is one of the highest among the monogenic neurological disorders in the developed world affecting 10–12 in 100 000 people (5). In HD there is a gain-of-function of the muHTT protein (6), while the loss of wild-type HTT (wtHTT), to certain extent, was proven to be safe (7,8). Complete loss of

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wtHTT during embryonic development is lethal (9), while decreasing wtHTT levels in adult animals seems to be well tolerated (10,11). Encouragingly, in a human embryonic stem cell-derived neuronal model, a 10–20% reduction of the muHTT alone was sufficient to show a significant reduction of toxicity, whereas reducing wtHTT by up to 90% was described as safe (12). In addition, studies in Rhesus monkeys showed that a 45% reduction in the putamen resulted in no mobility or neurotoxic effects during the 6 weeks follow up (13). Interestingly, humans with a heterozygous translocation interrupting the *HTT* gene and who have reduced levels of HTT displayed no phenotypic abnormality (5,7,8). Altogether, lowering muHTT at the expense of a partial loss of wtHTT may be acceptable in a clinical context for adult patients.

There is increasing evidence that mu*HTT* transcripts also contribute to HD toxicity (14,15). RNA-related toxicity mechanisms have not been equally explored as those related to the muHTT protein although both add to the overall pathogenic gain-of-function effect (16,17). CAG-expanded *HTT* transcripts were shown to be retained in the nucleus of human HD fibroblasts and to co-localize with a splicing factor involved in the pathogenesis of CTG/CAG expanded transcripts (18,19). Moreover, the CAG repeat transcripts were demonstrated to form secondary structures that can be cleaved by Dicer resulting in aberrant generation of short repeated RNA (20,21). The latter was proven to cause inherent toxicity in a neuronal cell model (14), potentially contributing to the disease phenotype.

The monogenic origin of HD makes it an appealing target using oligonucleotides (ONs) that can affect the *HTT* gene expression. Different ON strategies, indeed, have been applied for targeting *HTT* such as siRNAs (22), splice-switching ONs (23), single nucleotide polymorphism (SNP) - targeting ONs (24), zinc finger nucleases (25) and antisense ONs working via either RNase-mediated degradation (26) or steric blocking of the *HTT* mRNA. Previous trials for blocking *HTT* with ONs can be categorized, according to their objective, into two main classes: allele-specific and non-allele specific strategies. Allele-specific silencing has been successfully achieved via antisense ONs that can hybridize to the *HTT* mRNA and by steric blocking prevent its translation. Such ONs, due to the difference in stability possessed by the mutant and wild-type RNA structures, could bind better to the mu*HTT* mRNA (27). This approach was successfully achieved using antisense ONs with different chemistries (28–30), single-stranded RNAs (31,32) and siRNAs with mismatches (33–35), which improved the mutant selectivity over the wild-type. ONs working by this strategy will not decrease the *HTT* mRNA levels (29,33). Alternatively, when the CAG tract expansion is linked with SNPs that can be targeted by gene silencing ONs, allele-specific *HTT* reduction can be accomplished (36). SNP targeting was first achieved using siRNAs (37), and subsequently various antisense ONs with different chemical modifications have shown great success (38,39). To use this approach, prospective treatments need to be tailored to each group of individuals that carry the same SNP.

The non-allele specific approach has been recently suggested as a valid and safe option that would avoid the time-consuming and costly individualized therapy (40,41). Non-allele specific targeting has been achieved using antisense ONs targeting outside the trinucleotide repeat region and inducing degradation of *HTT* mRNA via RNase H recruitment (26). Furthermore, a number of siRNAs has been developed to efficiently degrade the *HTT* mRNA via the RNA interference process (42). In fact, non-allele specific down-regulation can be considered successful as long as the level of wtHTT remains above the threshold required for normal cell function (10,11,43).

We reasoned that if both polyQ-expanded HTT protein and its transcript are neurotoxic, achieving a specific down-regulation without involvement of RNA degradation, while keeping the wtHTT to the acceptable safe thresholds, could be an attractive therapeutic option. ONs were designed with the aim of binding to the *HTT* gene and block its transcription. Interfering at the DNA level would provide a partial, but potentially long-term down-regulation, which in turn could decrease the dose needed. We designed ONs as mixmers of DNA or 2′-*O*-methyl-RNA with locked nucleic acid (LNA) (44), which have shown previously to improve the capacity of strand-invasion into double-stranded DNA (dsDNA) (45), and in one construct also including novel 2′-glycylamino-LNAs.

In this work, we report an LNA/DNA ON-based strategy, where the drug successfully binds to the *HTT* gene template strand down-regulating mRNA and protein levels in HD patient fibroblasts. Reduction of HTT protein is shown to last for 10 days, which was the longest time assayed in the study. RNA polymerase II serine 2 phosphorylation is significantly impaired at the *HTT* gene supporting an ON effect on gene expression at the transcriptional level. In addition, we address the possibility to use cell penetrating peptide (CPP)-based delivery and also naked uptake, known as gymnosis (46), of the ONs, which can be of value for future *in vivo* applications.

MATERIALS AND METHODS

Oligonucleotides

LNA/DNA ONs were either bought from Eurogentec (Belgium) or synthesized at Nucleic Acid Center, University of Southern Denmark. CAG ONs were designed to target the DNA template strand while CTG ONs (13, 13gap and 19) were designed to target the mRNA and used as controls. CAG ONs were synthesized in different lengths ranging from 10- to 19-mers. The 12-, 13- and 14-mer CAG ONs were synthesized in both phosphodiester (PO) and phosphorothioate (PS) backbone format. The 14-mer CAG PS ON was synthesized either as an ON including 2′-*O*-methyl RNA /LNA, namely: CAG-14PS-OMe or as a CAG-14PS-gly ON where 5-methylcytosine-2′-glycylamino-LNA substitutes the regular cytidines (see Supplementary Methods for Synthesis Details (47)). A number of mismatched and scrambled ONs were used as controls (sequences found in Table 1). An siRNA targeting *HTT* transcripts outside the repeat region, and used previously (33,48), was obtained from Sigma. A complete list of the ON sequences can be found in Table 1.

Table 1. ONs used in the study. CAG ONs are designed to target the template strand of the *HTT* gene while CTG ONS are designed to target the mRNA and used here as controls. All the ONs used in the study have PS backbone. The CAG-12, -13 and -14 are made in two different versions, one with PO and the other with PS backbone. LNA bases are written in capital letters while DNA in small. In the ON CAG-14PS-OMe, the 2′-*O*-methyl RNA bases are superscripted with 'm'. In the ON CAG-14PS- gly, the 2′-glycylamino 5′-methylcytosine LNA nuccleotides are superscripted by 'gly'

ONs	Sequence
CAG-10PS	5′-cAgCAgCAgC
CAG-12PS and CAG-12PO (both Cy5 labelled)	5′-cAgCAgCAgCAg
CAG-13PS and CAG-13PO (both Cy5 labelled)	5′-cAgCAgCAgCAgc
CAG-14PS and CAG-14PO (both Cy5 labelled)	5′-cAgCAgCAgCAgCa
CAG-14PS-OMe	5′-mcAmgCAmgCAmgCAmgCma
CAG-14PS-gly	5′-cAgCglyAgCglyAgCglyAgCglya
CAG-15PS	5′-cAgCAgCAgCAgCAg
CAG-16PS	5′-cAgCAgCAgCAgCAgc
CAG-18PS	5′-cAgCAgCAgCAgCAgCAg
CAG-19PS	5′-cAgCAgCAgCAgCAgCAgc

Control ONs	Sequence
Scramble ON1 (scramb.1)	5′-gACgACgACgACgA
Scramble ON2 (scramb.2)	5′-gAcGAcGAcGAcGA
Mismatched ON1 (mismatch.1)	5′-aAgAAgAAgAAg
Mismatched ON2 (mismatch.2)	5′-aAgAAgAAgAAgAA
Mismatched ON3 (mismatch.3)	5′-cAaCAgCAgCAaCAgCAa
Control CTG-13PS	5′-cTgCTgCTgCTgc
Control CTG-13PS gap	5′-gcTgcTcgTgcTg
Control CTG-19PS	5′-gcTgcTgcTgcTgcTgcTg
siRNA	5′-CAGACAAUGAUUCACACGG[dT][dT] [dT][dT]UUCUGUUACUAAGUGUGCC

Cell culture, transfections and gymnosis

Huntington patients' primary fibroblasts GM04281, GM09197 and GM04022 were obtained from the Coriell Cell repositories. GM04281, GM09197 and GM04022 HD fibroblasts in this order have numbers of 68/17, 151/21 and 44/12 trinucleotide repeats representing the mutant/wild-type HTT, respectively. Fibroblasts were grown in Dulbecco's Modified Eagle Medium with L-glutamine, pyruvate and low glucose (Invitrogen, Sweden), supplemented with 10% fetal bovine serum (Invitrogen). Fibroblasts were maintained at 37°C, 5% CO_2 in humidified incubators. One day prior to transfection, fibroblasts were seeded at 30 000 cells per well in a 24-well plate or at 160 000 cells per well in a 6-well plate. ONs were formulated either with lipofectamine RNAiMAX® (RNAiMAX) according to the manufacturer's protocol or with the CPP Pepfect 14 (PF14) in a molar ratio 1:5 (ON: peptide). Formulations were added to the fibroblasts, while still growing in 10% fetal bovine serum medium, to give a 100 nM final concentration of the ON. Gymnosis was performed by adding the ONs directly to the cells one day after seeding without using transfection reagents. Unless otherwise stated, cells were cultured for four days following transfections or kept under gymnosis for four days, after which the cells were lysed for RNA or protein assessment. For the 7- and 10-day cultures, more medium was added to the cells.

Fluorescence microscopy

Cy5 labeled ONs were transfected into GM04281 fibroblasts at 100 nM concentration using RNAiMAX. Before imaging, the medium was aspirated and cells were carefully rinsed using fresh full medium to ensure the removal of any ON or formulation residuals. Live cells were then imaged using a fluorescence microscope (Olympus IX81, Olympus America Inc.) and the signal of the Cy5-labeled ONs was detected.

RNA isolation and quantitative reverse transcriptase multiplex PCR (qRT-PCR)

At specific time points, cells were lysed and total RNA isolated using the RNeasy plus kit (QIAGEN, Sweden). RNA was analyzed using multiplex q RT-PCR to amplify both *HTT* and *HPRT1* as an endogenous control. This assay was performed using the Quantifast® Multiplex RT-PCR kit (Qiagen). Sequences of primers and probes for *HTT* and *HPRT* (Sigma) were as follows: HTT-fwd: 5′- gactcgaacaagcaagag, HTT-rev: 5′-gcctttaacaaaaccttaatttc, HPRT-fwd: 5′- gagctattgtaatgaccagtc, HPRT-rev: 5′-tgaccaaggaaagcaaag, HTT taqman probe: 5′-[JOE]gaagaatcagtccaggagacc[BHQ1] and HPRT taqman probe: 5′- [6FAM]tgccagtgtcaattatatcttccacaa[BHQ1] where JOE and 6-FAM are two fluorophores having different emission spectra and with Black Hole Quencher (BHQ1) used as quencher. Multiplex qRT-PCR reaction setup was done according to the Quantifast® kit protocol, where 35 ng of RNA was used for all reactions and the final volume of each reaction was 25 μl. Standard curves were made using known amount of RNA and serially diluted in order to confirm the efficiency of PCR, which was close to 100%. Cycling conditions of PCR were: 20 min 50°C for reverse transcription, 5 min 95°C for PCR initial activation step and 45 cycles, each of 2 steps: 15 s, 95°C denaturation and 30 s, 60°C for annealing/extension. Quantitative RT-PCR was performed using the StepOnePlus® Real time PCR system (Applied Biosystems, Sweden) and the data

were analyzed by the $\Delta\Delta C_t$ method using the StepOne® software version 2.2.

Western blotting

Cells in the 6-well plates were lysed using a mixture of RIPA lysis buffer and NuPAGE® LDS sample buffer (Invitrogen) in a ratio of 3:1, respectively. The plates were kept on ice for 30 min, and then the lysates were transferred to Eppendorf tubes, which were shaken for 30 min at 4°C to ensure cell lysis. Four microliters of NuPAGE® sample reducing agent 10x (Invitrogen) were added to 40 μl cell lysate and the mixture was heated to 95°C for 5 min just before loading on the gel. Proteins were separated on NuPAGE® 3–8% tri-acetate gels at 70 V for 25 min followed by 125 V for 6 h and the whole electrophoresis procedure was performed on ice at 4°C. The gels were then transferred onto nitrocellulose membranes using the IBlot® system (Invitrogen) and subsequently the membranes were blocked with Odyssey Blocking Buffer (LI-COR Biosciences GmbH) for 1 h. Membranes were probed using anti-HTT primary antibody (MAB2166, Millipore) and anti-importin 7 antibody, which was used as a housekeeping control, diluted at 1:500 and 1:1000, respectively, over night. Signals were detected after soaking with secondary antibodies, which were goat anti-mouse and goat anti-rabbit for HTT and importin 7, respectively. For checking the expression of control genes containing CAG repeats, NuPAGE® Bis-Tris 4–12% gels were used to separate the proteins. After transfer and blocking as described, the membranes were probed with monoclonal Anti-TBP (Tata box binding protein) (Sigma) 1:2000, polyclonal Anti-FOXP2 (Forkhead box protein P2) (Abcam) 1:1000 and polyclonal Anti-ATN1 (Atrophoin-1) (Santa Cruz) 1:1000. All Western blot signals were scanned using the Odyssey Imager (LI-COR Biosciences GmbH).

Thermal UV-melting experiments

All *T*m experiments were performed using a Varian Cary 300 UV-VIS spectrophotometer. The ON concentrations used were 3 μM each in a total volume of 400 μl containing intra-nuclear salt buffer (Tris-acetate 50 mM (pH 7.4), 120 mM KCl, 5 mM NaCl, 0.5 mM Magnesium acetate). The melting profile started at 95°C and was followed down to 20°C at 0.2°C/min, and returning to 95°C at the same rate. Absorbance was recorded at 260 nm and *T*m calculated from processed curves using the instrument software algorithms.

Plasmid cloning and hybridization experiments

Plasmid 1735-1 contains a 72 CAG repeat sequence (216 bp) and was generated by Mutagenex using standard molecular cloning procedures. The control plasmid contains the same backbone, but lacks the trinucleotide repeat region. ONs and plasmids were hybridized for 72 h at 37°C in 10 μl using 500 ng of plasmid and 40 pmol of ON in intra-nuclear salt buffer, unless otherwise stated.

Restriction enzyme assay of ON target binding

Restriction enzymes AvrII (#FD1564) and HindIII (#FD0504) (Thermo Scientific) were incubated with 500 ng plasmid 1735-1 (pre-incubated 24 h with or without 4 μM CAG-ONs), according to the manufacturer's protocol. The samples were analyzed by polyacrylamide gel electrophoresis using 20% polyacrylamide gels in TBE (Tris borate ethylenediaminetetraaceti cacid) (Life Technologies). An amount of 200 ng of DNA in DNA Loading Dye buffer (Thermo 6x R0611 diluted to 1x in ddH_2O) was loaded into each well. Electrophoresis was performed at 110 V for 1.2 h in 1xTBE buffer. The gel was stained using 1xSYBRGold nucleic acid gel stain (Invitrogen #S-11494) for 7 min on shaker, visualized using a VersaDoc system (BioRad) and analyzed by the QuantityOne software (BioRad).

Chromatin Immunoprecipitation (ChIP-qPCR)

ChIP-qPCR was performed using the iDEAL ChIP-seq kit for histones (Diagenode) according to the manufacturer's instructions. Briefly, cells (transfected with either CAG-19PS or scrambled ON2) were fixed in 1% formaldehyde for 8 min, and following chromatin extraction aliquots of one million cells, were sonicated for 12 cycles of 30 s on/off in 1.5 ml Bioruptor Microtubes (Diagenode) 1.5 ml tubes with a Bioruptor pico (Diagenode). One million cells were used per immunoprecipitation with 3 μg of antibodies against active histone modifications: histone 3 lysine 4 trimethylation (H3K4me3) (Ab-003-050, Diagenode) and histone 3 lysine 27 acetylation (H3K27ac) (ab4729, Abcam) or 5 μg of RNA Pol II ser2 antibody (ab5095, Abcam). DNA was isolated following reverse cross-linking with QIAquick PCR Purification Kit (QIAGEN). ChIP DNA was diluted for qPCR fifty times. Briefly 3 μl of diluted ChIP DNA was used per reaction, in triplicate with FastStart Universal SYBR Green Master (Roche). Primers are listed in Table 2.

STATISTICS

Data are expressed as mean ± SEM. Statistical analyses were performed using Student's *t*-test for comparison of means. A probability of less than 0.05 was considered to be statistically significant.

RESULTS

CAG PS ONs significantly down-regulate *HTT* gene expression on both mRNA and protein levels

The approach taken was to generate a set of short LNA–DNA mixmers directed against the transcribed repeat region in the DNA duplex with the aim of reducing the *HTT* mRNA. LNA/DNA CAG ONs (12-, 13- and 14-mers) were first designed so that they bind via complementarity to the *HTT* DNA template strand. Three of the ONs were synthesized with a PO backbone, while the other three were PS ONs. In order to test the efficiency of the ONs, we first used the lipid transfection reagent RNAiMAX as a delivery vehicle to target primary HD patient fibroblasts GM04281. Prominent and reproducible HTT knockdown on both mRNA (Figure 1A) and protein levels (Figure 2)

Table 2. ChIP-qPCR primers used in the study

Primer #	Location	Forward sequence	Reverse sequence
1	HTT TSS	GGTTCTGCTTTTACCTGCGG	CTCGGGCCGACTCGC
2	Exon 1	GGTCCAAGATGGACGGC	AGCACCGGGGCAATGAAT
3	5′ of CAG repeat	ATTGCCCCGGTGCTGAG	GGACTTGAGGGACTCGAAGG
4	Gene body (intron 1)	GCTCCCTCACTTGGGTCTTC	CAAGTTCTCGCCCCAACTCT
5	Gene body (intron 1)	GTCAGGCTTGCCAGAATACG	TGGGGTTCCGCTAGATGTTT
6	Gene body (intron 1)	GAAGACCTTTCTGCTGGGCT	TCTCCTTTGTCAAGGCAGCAA
7	Gene body (intron 1)	TTCCTATCTGGTGTTTCCCTGAC	TTAACACTCGATTAACCCTGACA
8	Gene body (intron 1)	TGAGTAAAGACCTCAAGCGAGT	GAAGATTTTGGACCTGTTCCCCC
-	Exon 30	TGGGGACAGTACTTCAACGC	ACCTTGAAAATGTTTCTTCTGGCA
-	Exon 67	TCATCAGCAGGATGGGCAAG	AGTCAGCAGCCGGTGATATG

could successfully be achieved using the PS ONs. The efficiency of the PS ONs was significantly higher than that obtained by the PO ONs. In order to investigate if the uptake mechanism could be an explanation for such pronounced difference in activity, Cy5-labeled ONs of either PS or PO backbone chemistry were transfected followed by imaging of the live cells by fluorescence microscopy 4 days after transfection. The PS ONs showed distinct nuclear localization, while the POs were mainly seen in the cytoplasm (Figure 1B). When HTT protein levels were monitored 7 days after a single-dose transfection, the same knockdown efficiency of the PS ONs could still be observed (Figure 2C and D). Control ONs (mismatched ON1 and mismatched ON2) did not cause any significant reduction in *HTT* mRNA (Figure 1A) or HTT protein levels (Figure 2).

Effect of the ON length on the *HTT* knock-down efficiency and allele selectivity

We next wanted to test how changing of the CAG PS ON length would affect HTT down-regulation. Shortening the ON into a 10-mer completely abolished the effect, while ONs of 15-, 16-, 18- and 19-mer length significantly reduced *HTT* mRNA to levels, similar to the previously evaluated 12-, 13- and 14-mers (Figure 3A). The percentage of remaining *HTT* mRNA ranged from 35% to 50%. Reduced HTT protein levels were observed 4 days after transfecting HD patient fibroblasts with the CAG PS ONs (Figure 4A and B). The reduction in HTT protein levels was significant compared to all the control ONs tested in the same experiments. A dose response curve was made to one of the ONs (CAG-16PS) both for mRNA and protein levels (Supplementary Figure S1). Moreover, the knockdown effect on protein levels lasted for 7 days after single-dose transfections (Figure 4C and D). There was no significant difference between the effects on the muHTT versus wtHTT protein levels using the shorter ONs. However, the longest ON used in the study (CAG-19PS) showed significantly higher reduction of the muHTT protein as compared to the wtHTT after both 4 and 7 days (Figure 4B and D). To further investigate whether the effect starts at earlier time points and if it lasts longer, additional time-points were included in the study showing that the down-regulation of *HTT* mRNA started 1 day post transfection (although the effect was weaker and significantly different from that obtained after 4 days). Efficiency of the ONs even remained up to 10 days, which was the longest time tested in the study (Supplementary Figure S2). The selected CAG ONs were also tested in two other HD fibroblast cell lines: one with a higher repeat number (151 mu/21 wt): GM09197 and the other with lower number (44 mu/12 wt): GM04022. Interestingly, the selected CAG ONs showed efficiency in both cell lines similar to that obtained in the GM04281 (Supplementary Figure S3).

CAG ONs do not affect the expression of other CAG repeat containing genes

A number of other genes are known to contain tracts of CAG repeats (27,49). In order to evaluate the potential for 'off-target' effects of the examined CAG ONs, we checked the expression levels of control genes after treating the GM04281 HD fibroblasts. Western blot analysis showed no effect for CAG ONs compared to scrambled ONs on the expression levels of TBP, which contains CAG repeat tracts of up to 19 repeats in the coding region, FOXP2, containing a mixed stretch of 40 CAG and CAA repeats (50), or on the levels of ATN1 having 15 CAG repeats (Supplementary Figure S4).

CAG ONs down-regulate *HTT* mRNA more potently as compared to CTG ONs

Knocking down *HTT* mRNA by a single-stranded ON can be achieved using either an ON that binds to mRNA and induces RNase H degradation or an ON that binds to DNA and blocks its transcription. To exclude that the *HTT* mRNA down-regulation effect obtained here could be due to mRNA binding, we compared our CAG ONs side by side to ONs complementary to the *HTT* mRNA, i.e. CTG ONs. Two 13-mer LNA/DNA CTG control ONs: CTG-13PS and CTG-13PS gap (Table 1), which differ in the distribution of LNA and DNA base composition, were designed and tested in GM04281 HD fibroblasts. None of the control CTG ONs caused any reduction of the *HTT* mRNA as compared to the CAG-13PS, which significantly did (Figure 3B). A 19-mer LNA/DNA CTG ON (control CTG-19PS) resulted in some reduction in *HTT* mRNA (35%), as was previously reported (27), and further discussed below. Thus, the remaining *HTT* mRNA level after transfection of control CTG-19PS (65%) was significantly higher than that observed after transfection of CAG-19PS (43%) (Figure 3B). As expected, and as reported (27), transfection with the control CTG-19PS ON caused a marked reduction of HTT protein, with a significantly enhanced effect on the mutated protein after both 4 and 7 days (Figure 5). This observation indicates that the investigated CAG ONs, which

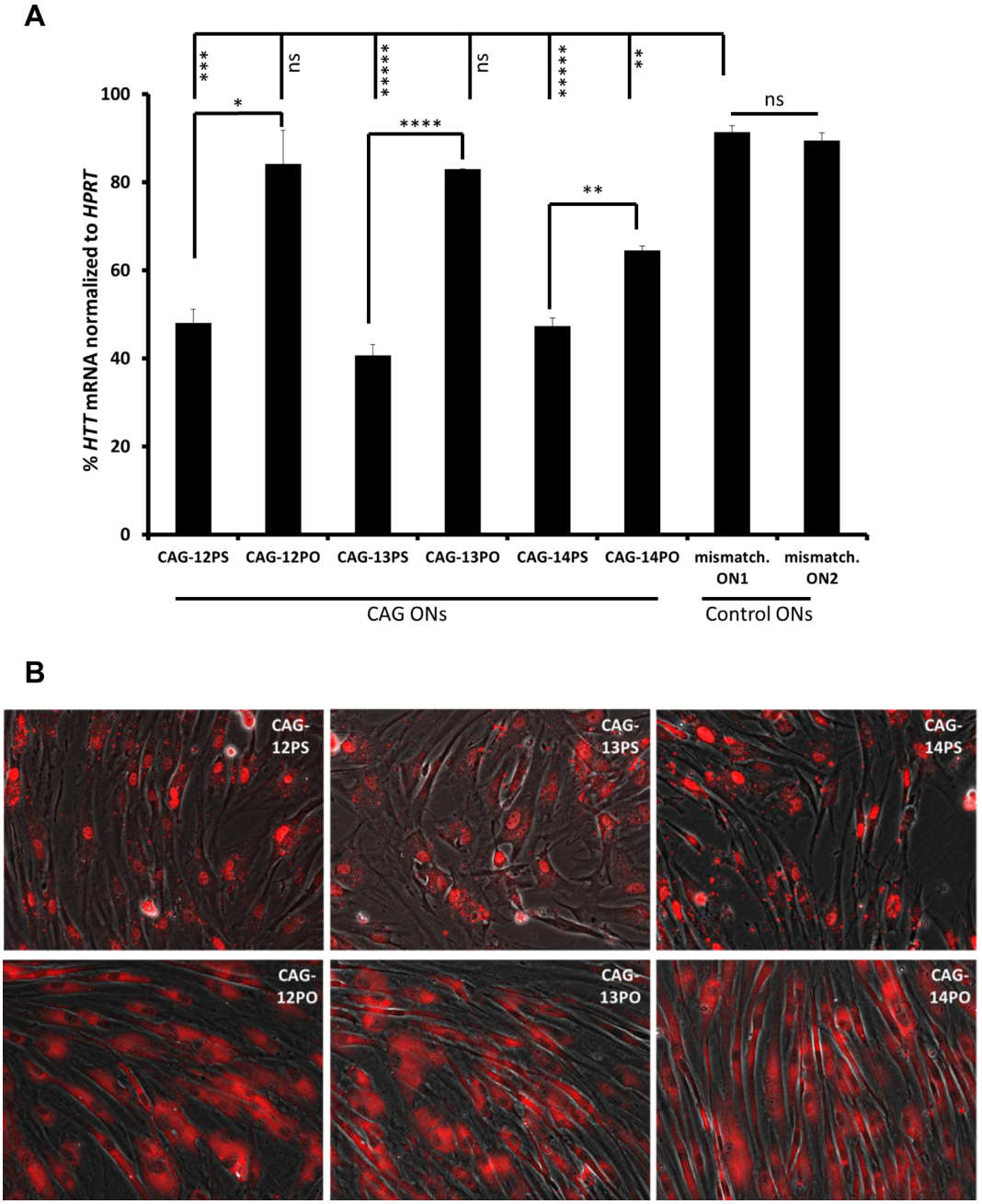

Figure 1. CAG phosphorothioate (PS) oligonucleotides (ONs) reach the nucleus and significantly down-regulate Huntington (*HTT*) gene expression on mRNA level. LNA/DNA CAG 12-, 13- or 14-mer ONs, with either PS or PO backbone, were transfected at 100 nM concentration after formulation with the commercial cationic lipid RNAiMAX into human HD patient fibroblasts GM04281. Four days post treatment, the cells were lysed and RNA was isolated and further analyzed by quantitative reverse transcriptase multiplex PCR (qRT-PCR). (**A**) Shows the *HTT* mRNA levels after each treatment normalized to *HPRT* as a housekeeping reference gene. Significant differences were found between CAG-12PS versus CAG-12PO ($P < 0.05$), CAG-13PS versus CAG-13PO ($P < 0.0001$) and CAG-14PS versus CAG-14PO ($P < 0.01$). Mismatched ON1 and mismatched ON2 were used as control ONs and were significantly different from all constructs except for the 13PO and 14PO (significance levels shown in the figure). (**B**) Shows the uptake behavior of Cy5 labelled ONs (PS or PO backbone) after transfection into GM04281 fibroblasts as detected by fluorescence microscopy of live cells.

down-regulate *HTT* on the mRNA level, are working via a mechanism that does not involve binding to mRNA, but is instead compatible with an effect caused by binding to the *HTT* gene.

UV melting measurements

To further confirm that the CAG ONs do not bind to the RNA sense sequence, *T*m was measured for the combination of CAG-13PS and RNA (19-mer CAG ON) and compared to that of control CTG-13PS and RNA. The RNA used as target was a 19-mer CAG ON and the analysis

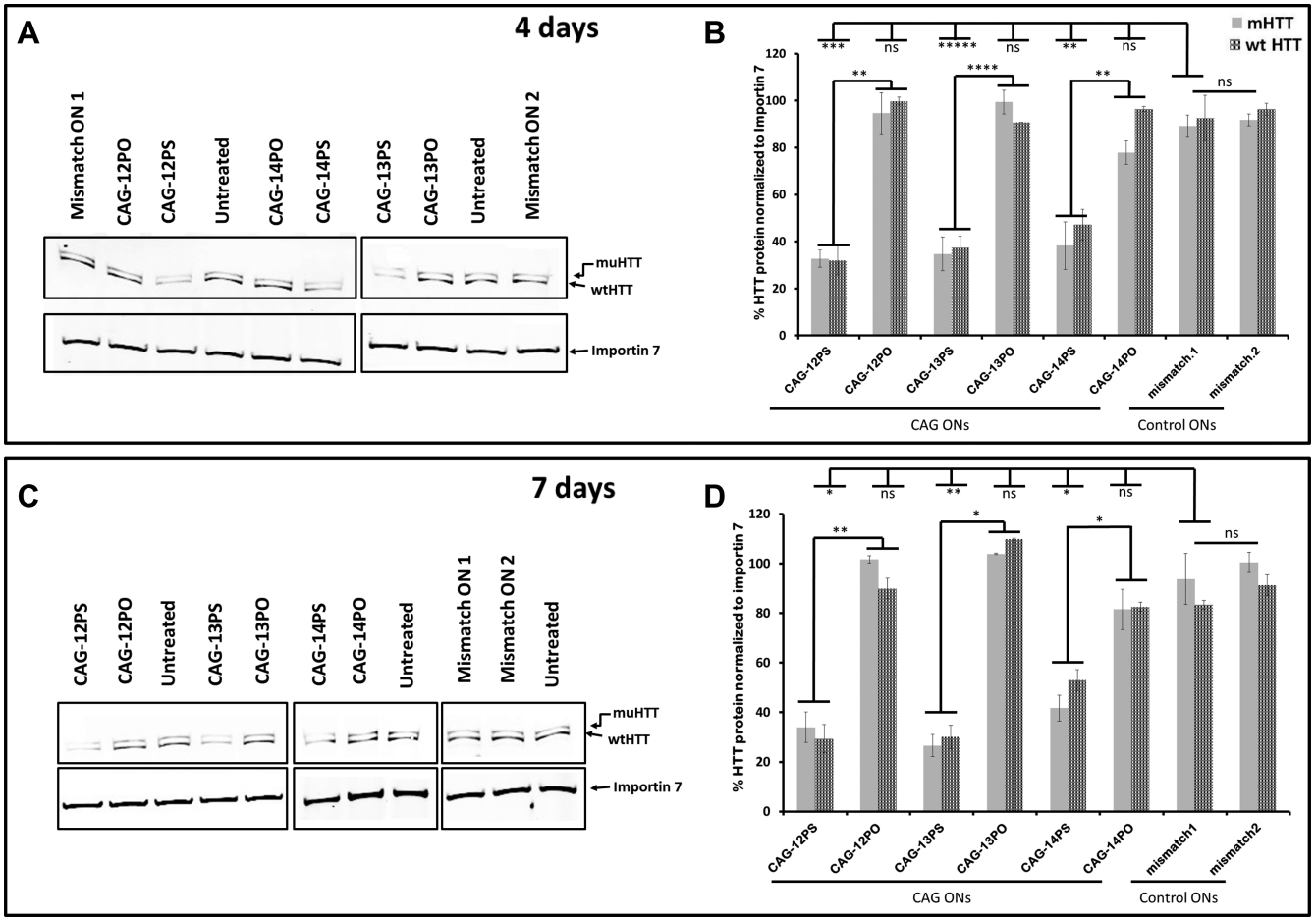

Figure 2. CAG PS ONs significantly down-regulate *HTT* gene expression on protein level. LNA/DNA CAG 12-, 13- or 14-mer ONs, with either PS or PO backbone, were transfected at 100 nM concentration after formulation with RNAiMAX into human HD patient fibroblasts GM04281. (**A** and **B**) Four days or (**C** and **D**) seven days post treatment, cells were lysed and western blots were performed to detect the HTT protein with its two isomers: mutant HTT (muHTT) and wild-type (wtHTT). Importin 7 was used as a housekeeping control. (A) Shows a representative western blot gel for the 4 days-time point, while (**C**) shows a representative gel for 7 days. Results from three replicate experiments were quantified and plotted in (B) and (D) for the 4 and 7 days data, respectively. There were significant differences between the total (mutant + wild-type) remaining HTT protein amounts after the treatment with: CAG-12PS versus CAG-12PO (P <0.01 and P < 0.01, for 4 and 7 days), CAG-13PS versus CAG-13PO (P < 0.0001 and P < 0.05, for 4 and 7 days), CAG-14PS versus CAG-14PO (P < 0.01 and P < 0.05, for 4 and 7 days). Mismatched ON1 and ON2 were used as controls and there was no significant difference between them, whereas total HTT protein levels obtained by all PS ONs were significantly different from that obtained by mismatched ON1 (significance levels shown in the figure).

showed no evidence of any interaction between the CAG-13PS and the RNA, since there was no shift in the *T*m curve (Figure 6E). On the other hand, control CTG-13PS combined with the RNA target sequence markedly shifted *T*m > 20°C indicating binding and interaction on the RNA level (Figure 6C). In addition, the CAG-19 ON showed no binding to the RNA ON (Figure 6G). Due to the large shift in *T*m (>95°C) that the control CTG-19 ON together with the RNA would generate, this combination was never tested. Of the different ONs, only the target 19-mer CAG RNA ON alone (Figure 6A) showed an increased *T*m (around 62°C), indicating self-interactions not present in the CAG-13, CAG-19 or control CTG-13 ONs.

CAG ONs bind to and invade dsDNA as evidenced in a restriction enzyme assay

To further investigate the proposed dsDNA strand-invasion mechanism, we set out to test if the CAG ONs could bind to and invade supercoiled dsDNA. To study this, we used restriction enzymes cleaving near the ON binding sites. The change in the cleavage pattern resulting from ON-mediated steric blocking of enzyme activity has previously been described in the context of triple-helix formation (51,52). Plasmid 1735-1, which contains a repeat region consisting of 72 CAG triplets, was hybridized to CAG ONs (13-, 14-, 15- or 19-mers) for 72 h at 37°C. This plasmid contains AvrII and HindIII restriction enzyme sites, which were used in combination to linearize the plasmid followed by analysis using gel electrophoresis (Figure 7). HindIII cleaves the plasmid at two sites, one located 40 bp upstream of the trinucleotide repeat region and the other 742 bp 3′ of this region. AvrII

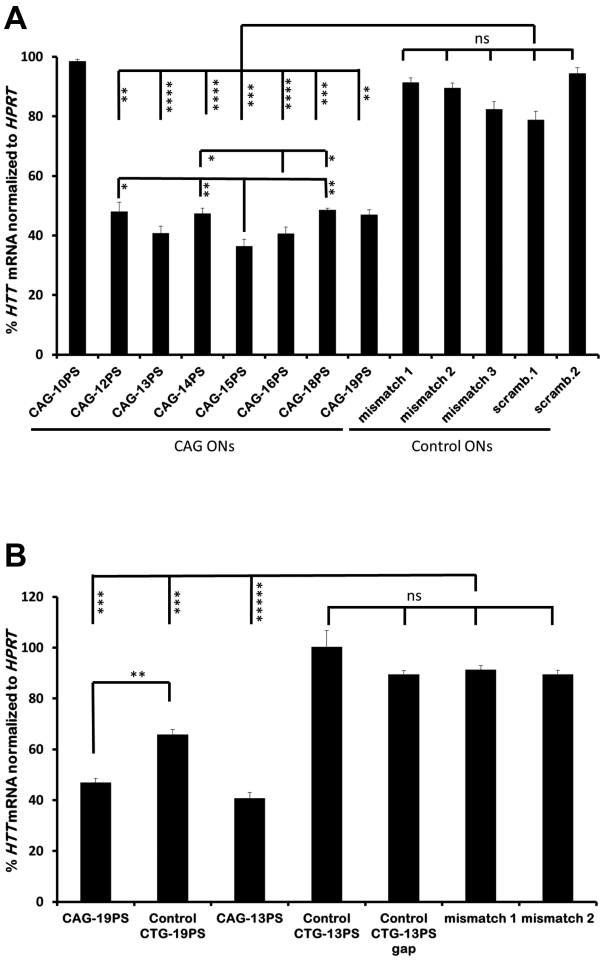

Figure 3. (**A**) Effect of the ON length on the *HTT* mRNA knockdown efficiency. LNA/DNA CAG 10- to 19-mer PS ONs were transfected at 100 nM concentration into GM04281 human HD fibroblasts using RNAiMAX. Four days post treatments, cells were lysed and RNA was isolated and analyzed by qRT-PCR using primers and probes for *HTT* and for *HPRT* as a housekeeping reference gene. *HTT* mRNA levels after treatment with CAG-12, -13, -14, -15, -16, 18- and -19 were all significantly reduced as compared to CAG-10PS and to all the mismatched and scrambled controls (significance levels between each ON and the scrambled ON1 are shown in the figure). CAG-15PS differed significantly as compared to CAG-12PS, CAG-14PS and CAG-18PS. CAG-16 PS differed also significantly as compared to CAG-14PS and CAG-18PS. No other significant differences were found between the ONs from 12 to 19-mer in *HTT* mRNA knockdown levels. (**B**) CAG ONs down-regulate *HTT* mRNA more potently as compared to control CTG ONs. The 13- and 19-mer ONs of either CAG or CTG sequence were transfected into HD fibroblasts GM04281 for 4 days and the remaining *HTT* mRNA levels were assessed using q RT-PCR. A significant difference was found between the remaining *HTT* mRNA levels after CAG-19PS versus control CTG-19PS treatment ($P < 0.01$) and they were both significantly different from the control ON (mismatched 1) ($P < 0.001$ and $P < 0.001$). CAG-13PS effect was significantly different from mismatched1 ON ($P < 0.00001$). CTG-13PS and CTG-13PS control ONs did not show any significant difference compared to the control mismatched ONs used in the study.

cuts at a site located only a single bp after the trinucleotide repeat region. Together they are expected to generate two fragments; however, as seen in Figure 7B, the cleavage by AvrII is hindered by the presence of a CAG ON. Moreover, the longer the CAG ON used, the less effective was AvrII digestion, as indicated by the weaker band generated with AvrII and the appearance of the band corresponding to HindIII cleavage only (Figure 7). Our interpretation is that this is due to the fact that a long CAG ON would have a greater probability to interfere with AvrII cleavage due to steric hindrance. The weaker bands seen in the gel (marked by a '*'), which also follow this pattern, we believe originate from a different plasmid sub-clone, which exists as a small fraction lacking a part of the 72 CAG region due to replication errors during plasmid preparation (Figure 7A).

CAG ONs significantly reduce serine 2 phosphorylated RNA Pol II occupancy across the *HTT* gene

In order to confirm the CAG ONs binding to chromosomal DNA, we set out to investigate first whether the reduction in *HTT* gene expression was associated with epigenetic inactivation of the *HTT* promoter or reduced promoter activity. We performed ChIP-qPCR for active histone marks H3K4me3 and H3K27ac, which are associated with the transcription start site (TSS) of actively transcribed genes (53). Primers were designed across the *HTT* gene 5′ region using ChIP-seq data from the ENCODE project to map to locations associated with active histone modifications across the *HTT* gene in normal conditions (Supplementary Figure S5A). No significant loss of either H3K4me3 or H3K27ac was observed after treatment with CAG-19PS compared to scrambled ON, suggesting the CAG ON's function to be independent of promoter activation and silencing (Supplementary Figure S5B and C). Given that the CAG ON targets the first exon of the *HTT* gene, downstream of the TSS and Pol II initiation site, we hypothesized that the CAG ON may influence Pol II elongation in transfected cells. This can be examined through measuring serine 2 (ser 2) phosphorylated RNA Pol II occupancy (54). Serine 2 phosphorylation of RNA Pol II occurs during productive elongation of Pol II following initiation and Pol II ser2 occupancy in the 3′ exons of genes is correlated to gene expression and elongation rates. PCR primer positions relative to RNA Pol II ser2 occupancy across the *HTT* gene in human cell lines is shown in Figure 8A. Interestingly, CAG-19PS did indeed reduce the 3′ occupancy of Pol II ser2 in the *HTT* gene, while showing a slight increase of Pol II at the 1st exon upstream of the transcribed trinucleotide repeat region (Figure 8B). It was observed that Pol II ser2 occupancy was higher upstream of the transcribed repeat region, but significantly reduced in the middle and 3′ end of the gene at exons 30 and 67 respectively. This provides proof that the mechanism of *HTT* mRNA reduction is mediated through a direct effect on transcription at the level of RNA Pol II elongation suggesting that binding of the ON in living cells poses a barrier to *HTT* gene transcription. No differences were observed at control loci in the last exons of the *PRDM1* or *ACTB* genes (Figure 8C).

CAG ONs can be delivered to patient fibroblasts by peptide and gymnotic delivery

PepFect 14 (PF14), a recently developed cell-penetrating peptide (CPP) (55), was tested as a delivery vehicle for the LNA/DNA CAG ONs. ON/PF14 formulations at molar ratio 1:5 resulted in similar activity as that obtained by

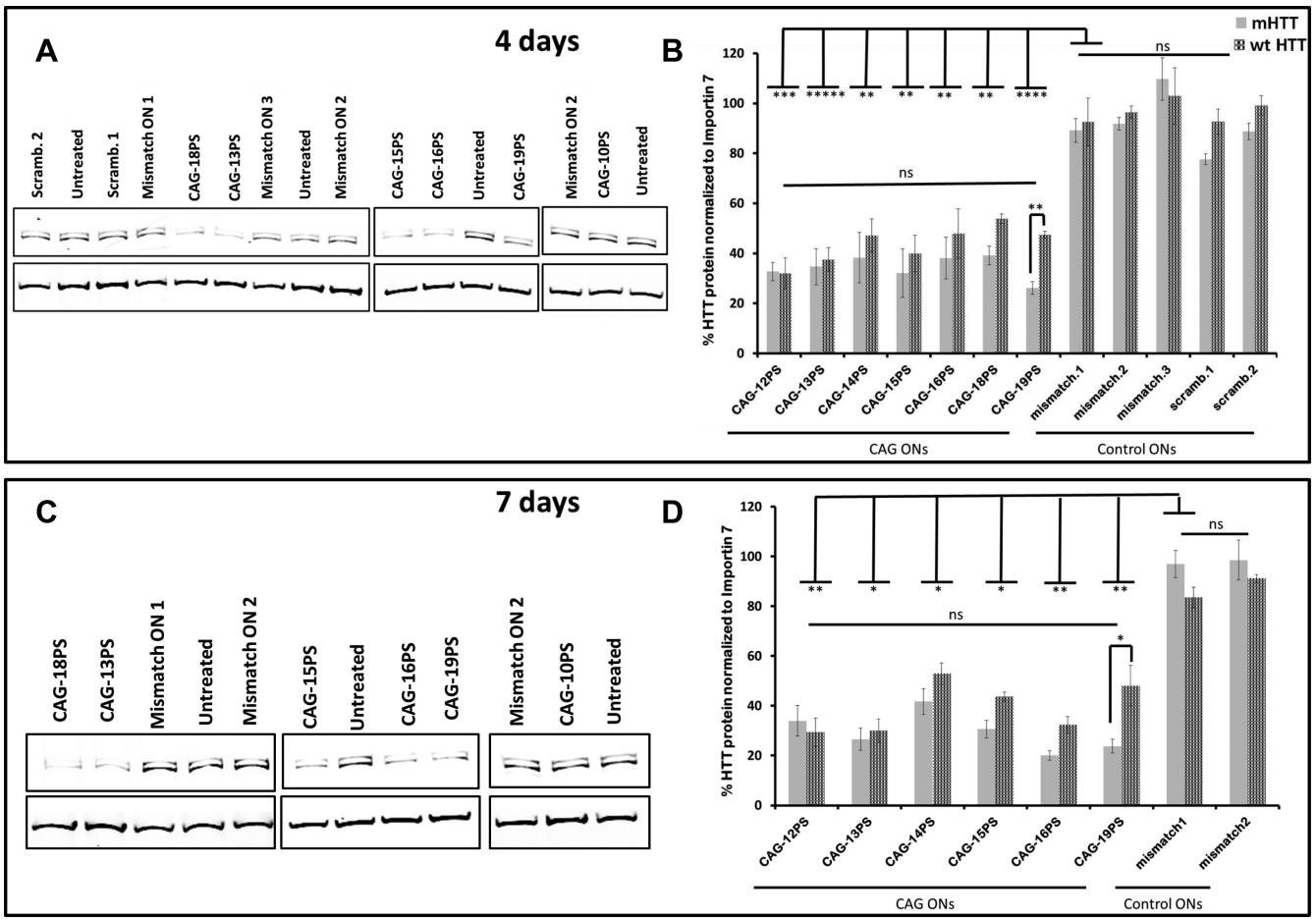

Figure 4. Effect of the ON length on the HTT protein knockdown efficiency and allele selectivity. LNA/DNA CAG 10- to 19-mer PS ONs were transfected at 100 nM concentration into GM04281 human HD fibroblasts using RNAiMAX. Four or seven days post treatments, cells were lysed and western blots were performed to detect the HTT protein with its two isomers: muHTT and wtHTT. Importin 7 was used as a housekeeping control. (**A**) Shows a representative western blot gel for the 4 days-time point, while (**C**) shows a representative gel for 7 days. Results from three replicate experiments were quantified and plotted in (**B**) and (**D**) for the 4 and 7 days data, respectively. Total HTT protein levels remained after treatments with ONs (from 12 to 19-mers) were significantly different from all control ONs tested in the study. Significance levels between each CAG ON and the control ON (mismatched 1) are shown in panel (B) and (D). No significant difference was found between the control ONs used in the study. A significant difference was found between the remaining muHTT and wtHTT protein levels 4 and 7 days after treatment with CAG-19PS ($P < 0.01$ and $P < 0.05$, respectively).

RNAiMAX formulations with CAG-13PS to 19PS (Figure 9A). PF14/CAG-12PS formulation was less efficient than that with RNAiMAX. Of great interest for CNS delivery is the ability of the ONs to enter the cells without any delivery agent, a phenomenon sometimes referred to as 'gymnosis' (46). ONs were added to the GM04281 HD fibroblasts at a concentration of 1 μM without using transfection reagents. Similar and significant levels of *HTT* mRNA knock-down was achieved with CAG-PS 13-, 14-, 15-, 16-, 18- and 19-mers when compared to control ONs while the 12-mer was less efficient (Figure 9A). Interestingly, the control CTG-19PS blocking effect on *HTT* mRNA could not be detected using PF14 or gymnotic delivery (Figure 9A), whereas, similar to the data presented in Figure 3B, RNAiMAX induced a reduction. An siRNA designed against *HTT* mRNA was used as a positive control for the down-regulation. The siRNA was highly efficient only after transfection reagent-aided delivery, but not in gymnosis. CAG-PS ONs (13- and 14-mers) showed markedly and significantly higher efficiency compared to PO ONs when formulated with PF14, similar to what was seen with RNAiMAX formulations (Figure 9B). Gymnosis of CAG-ONs having a PO backbone unexpectedly caused death of the fibroblasts grown for 7 days following the addition of the ONs. In contrast, the PS ONs were biocompatible, with fibroblasts showing no signs of toxicity by visual inspection (data not shown).

Effect of ON chemical modifications on efficiency

We finally wanted to assess how other types of chemical modification of the ONs could affect the silencing efficiency. CAG-14PS-OMe is a 14-mer CAG ON, with all DNA nucleotides exchanged for 2′-*O*-methyl RNA (Table 1). CAG-14PS-gly is another 14-mer CAG in which four LNA-based cytidines were exchanged by 5-methylcytosine-2′-glycylamino-LNAs (Table 1). Using RNAiMAX, parent CAG-14PS showed significantly better down-regulation efficiency than the two ONs with the modifications. However, gymnotic delivery of the three ONs at 1 μM gave the same activity (Figure 10A). We then generated a dose-response

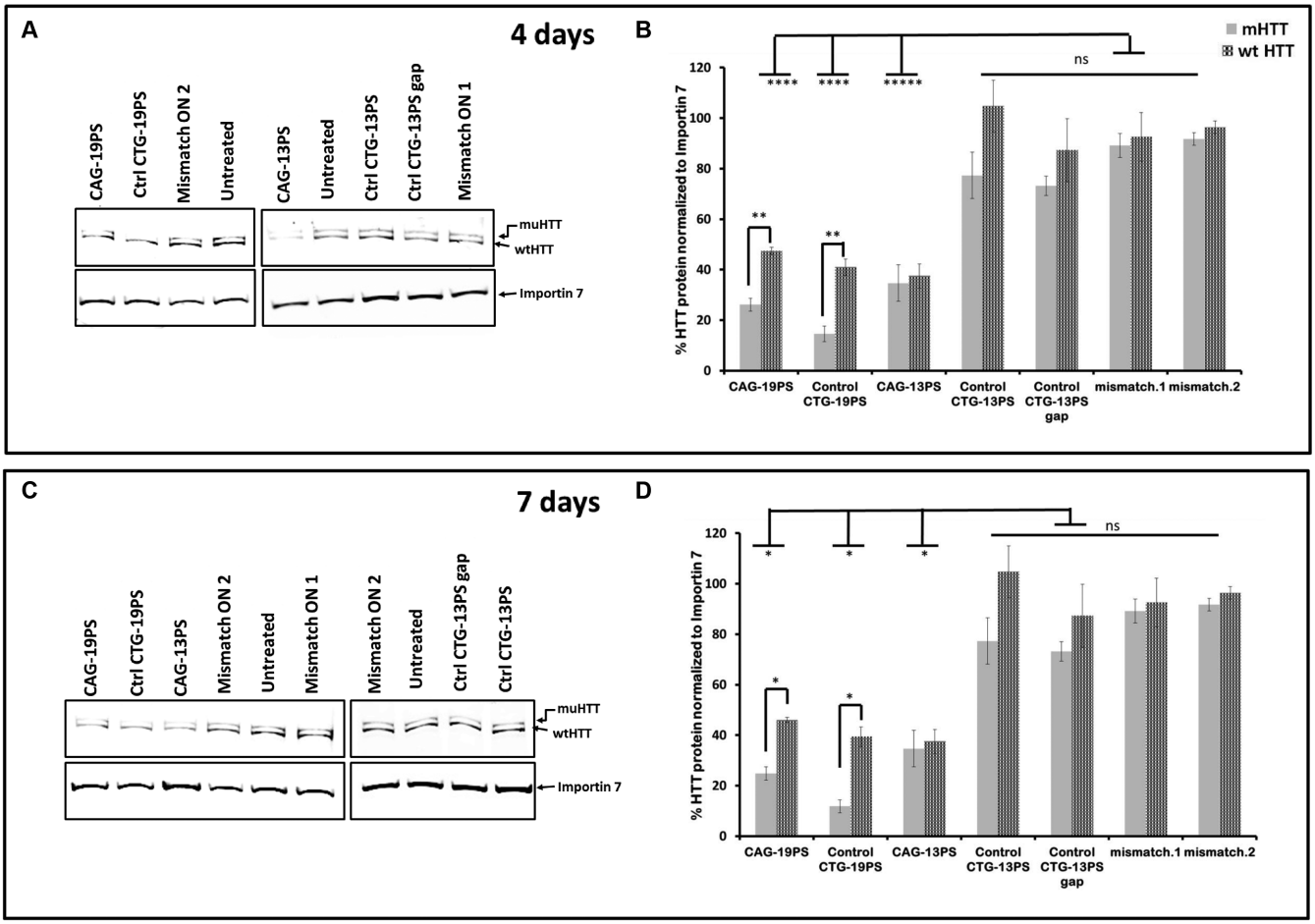

Figure 5. CAG ONs versus control CTG ONs in HTT protein down-regulation efficiency. The 13- and 19-mer ONs of either CAG or CTG sequence were transfected into HD fibroblasts GM04281 for 4 days or 7 days followed by cell lysis and western blots. (**A**) and (**C**) show representative western blot gels for the 4 and 7 days data, respectively. Quantification of western blot gels is shown in (**B**) for the 4 days and (**D**) for the 7 days results. Significant difference was found between the remaining muHTT and wtHTT protein levels 4 and 7 days after treatment with CAG-19PS (P <0.01 and P < 0.05) and with control CTG-19PS (P < 0.01and P < 0.05). No other significant difference was found between muHTT and wtHTT after treatment with any other ON. Total HTT protein was significantly reduced after treatment with CAG-19PS, control CTG-19PS and CAG-13PS as compared to the mismatched controls (significance levels between each ON and mismatched 1 are shown in the panels B and D). Control CTG-13PS and 13PSgap were not significant compared to the mismatched control ONs.

curve for gymnosis of the three ONs, which revealed that a 50% knocking down of *HTT* mRNA can be achieved after gymnosis at 0.5 μM of the parent 14-mer, while the two modified 14-mers need to be present at almost double this concentration in order to give a 50% reduction (Figure 10B). There were slight differences (not significant) in the dose-response curves, demonstrating that adding these modifications (in terms of type and number of modified bases) did not significantly improve the down-regulation efficiency of the CAG ON.

DISCUSSION

In this report, we are showing for the first time the possibility to down-regulate *HTT* on both mRNA and protein levels by single-stranded LNA/DNA ONs targeting the template strand of the trinucleotide repeat region. In contrast to antisense ONs targeting RNA, anti-gene ONs are designed for sequence-specific targeting of the chromosomal dsDNA. Such binding has previously been shown using triplex forming oligonucleotides (56) or by ONs with the ability to strand invade into dsDNA, as further discussed below. Since its development (57,58), and thanks to its great stability and high target affinity and recognition properties, LNA has been exploited for several applications including the design of antisense ONs working via different mechanisms (58). Furthermore, LNA-based ONs, namely Zorro-LNA and bisLNA, were proven to hold high capacity of strand-invasion into dsDNA (45,57,59–62). Short LNA/DNA ONs were also shown to bind as anti-gene agents and block gene expression (63).

We herein aimed at investigating the ability of short LNA/DNA ONs (ranging from 10- to 19- mers) to strand-invade into the *HTT* gene and down-regulate its level of expression. The ONs were designed with a CAG sequence, so that they would bind via complementarity to the CTG repeat sequence, i.e. to the template strand of the repeat region in the *HTT* gene. Since the anti-gene technology is far less explored in literature than the antisense, little is known

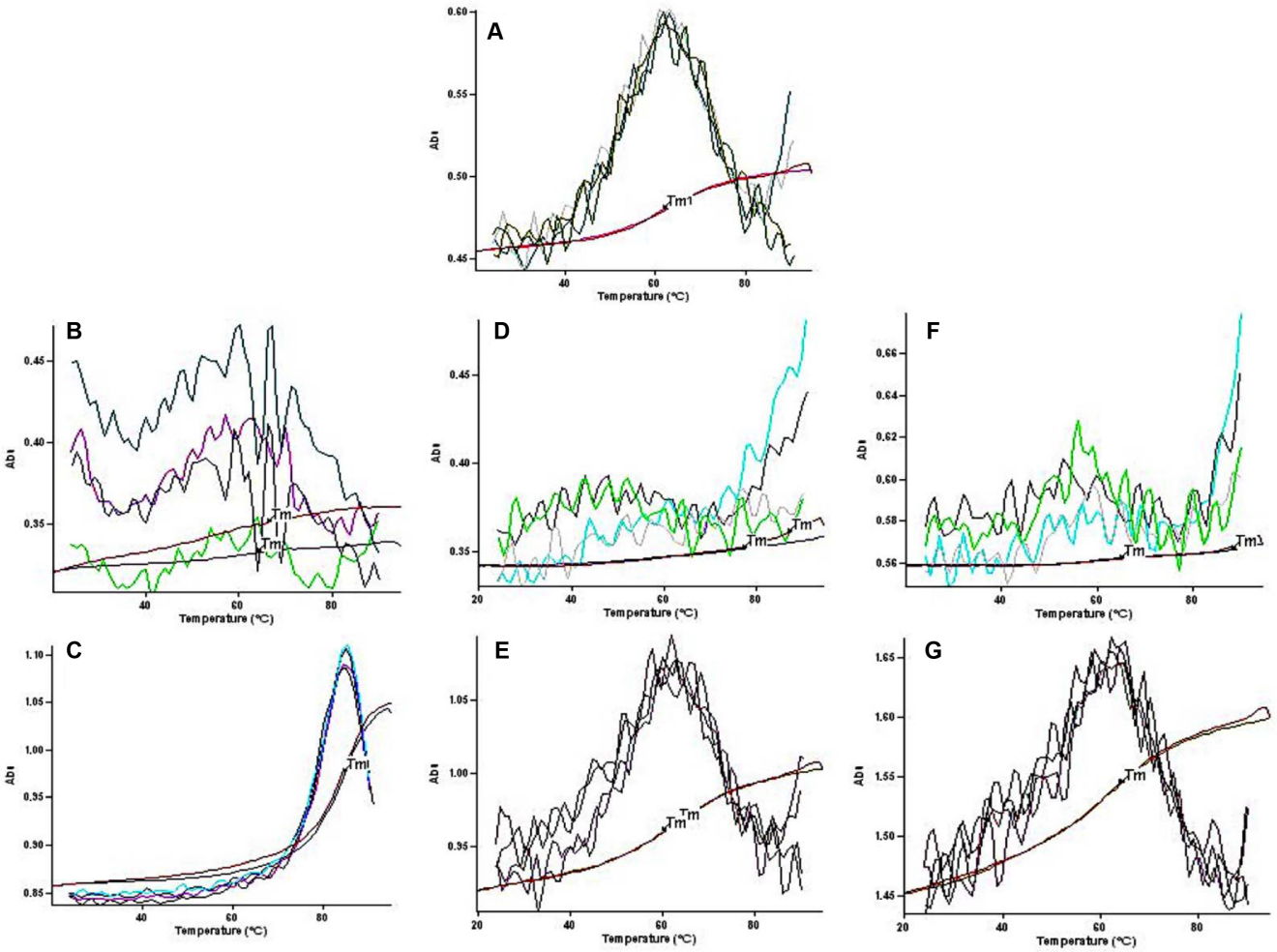

Figure 6. UV melting profiles of CAG, CTG and target RNA ONs. All graphs show the *T*m curves (marked 'Tm' in the figure) together with the second derivative in the background. (**A**) target RNA alone, (**B**) CTG13 alone, (**C**) RNA+CTG13, (**D**) CAG13 alone, (**E**) RNA+CAG13, (**F**) CAG19 alone and (**G**) RNA+CAG19. Only CTG13 is capable of binding the target RNA and shift the *T*m above the baseline of the RNA ON alone.

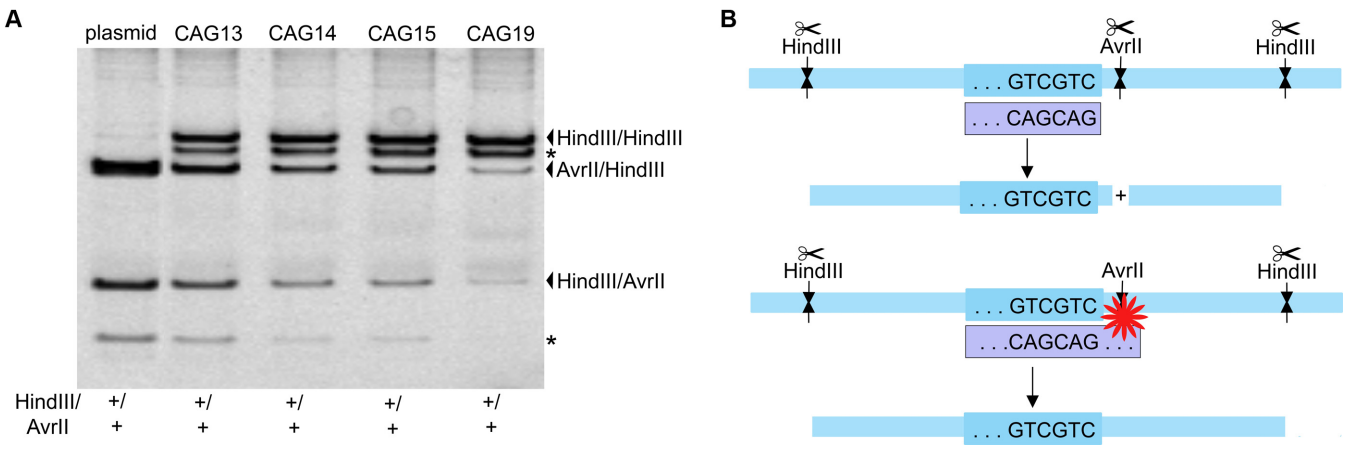

Figure 7. Binding of CAG ONs to target plasmid DNA visualized by restriction digestion and PAGE. Panel (**A**) shows the gel lanes with control and ON-hybridized plasmids digested with HindIII and AvrII, as indicated. * Denotes bands believed to originate from a subclone containing a truncated repeat region. Panel (**B**) schematically illustrates the mechanism by which the ONs hinder the cleavage by AvrII. Upper panel shows the cleavage pattern when no part of the binding ON is interfering with AvrII cleavage. Lower panel shows that longer ON binding randomly increases the probability of steric blocking of the Avr-site.

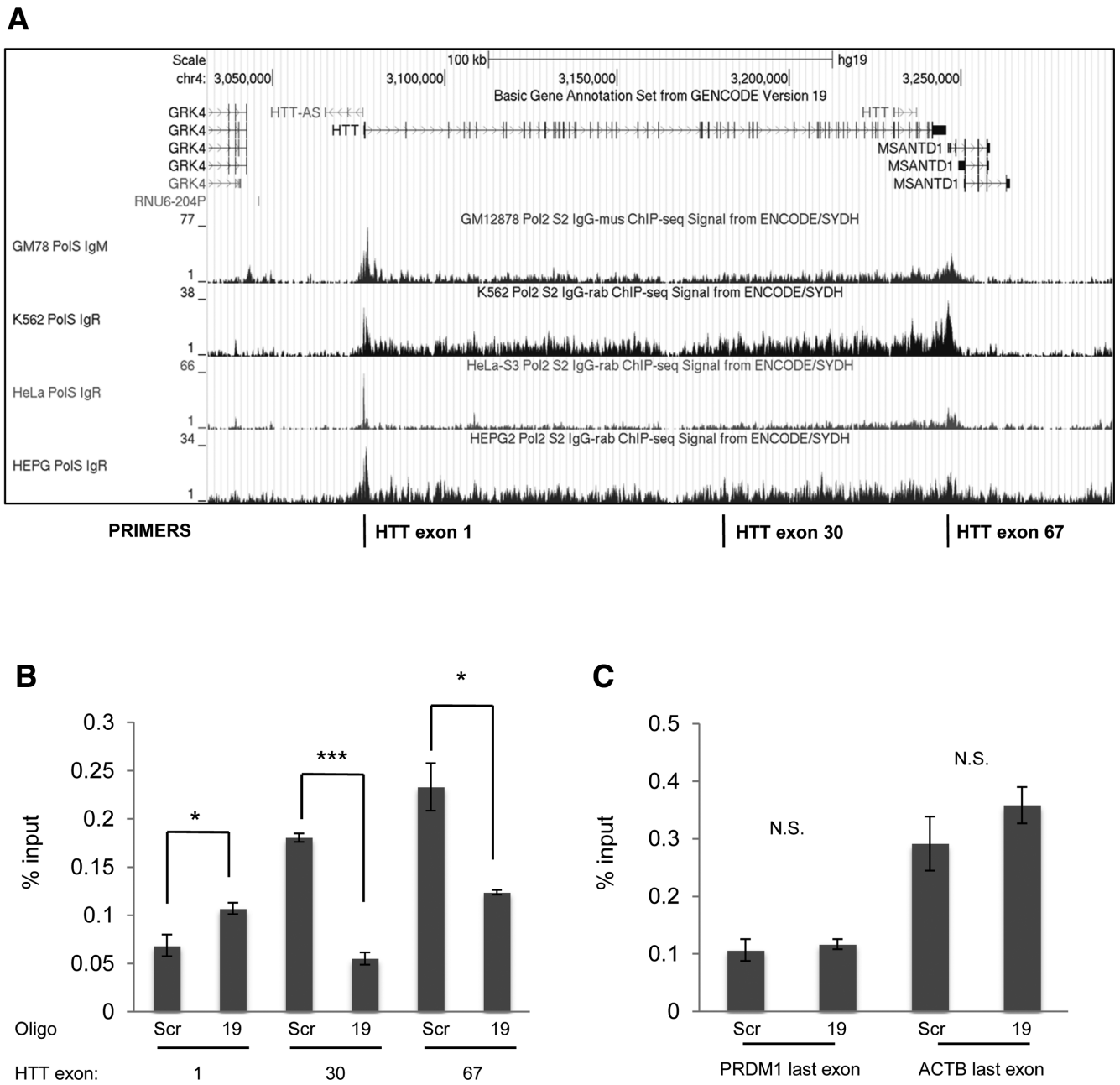

Figure 8. Serine 2 phosphorylated RNA Pol II occupancy across the *HTT* gene is reduced upon transfection with CAG-19PS (**A**) UCSC genome browser image demonstrating RNA Pol II ser2 occupancy across the *HTT* gene. Primer positions at exon 1 upstream of the CAG repeat and at exons 30 and the last exon, 67, are indicated. (**B**) RNA pol II occupancy is significantly increased at exon 1 of the *HTT* gene immediately upstream of the CAG repeat (*$P < 0.05$) and reduced at the 3′ exons 30 and 67 (*$P < 0.05$, ***$P < 0.001$). (**C**) No difference is observed at control loci in the last exons of the *PRDM1* or *ACTB* genes.

about how different ON structures and chemical modifications affect the binding efficiency.

Therefore, we investigated a few different types of ONs as well as various delivery methods. Collectively we found that the effect was not limited to a single ON chemistry or to a single delivery method. We started out by testing the two main backbone chemistries, commonly used for building antisense ONs: PO and PS backbones, and assessing the effect exerted by both in the anti-gene context. Interestingly, only transfection of PS ONs and not POs, resulted in a prominent and significant knocking-down of HTT mRNA and protein in HD patient fibroblasts. The fluorescence microscopy images displayed a marked nuclear uptake of the PS ONs versus the POs, which mostly stained the cytoplasm. Previous reports have demonstrated that PS ONs could bind to nuclear proteins (64,65), which would explain the uptake behavior observed here. Moreover, interactions of PS ONs with a number of other intracellular proteins have been recently addressed (66), suggesting that a PS backbone is required for the CAG ONs to be effective.

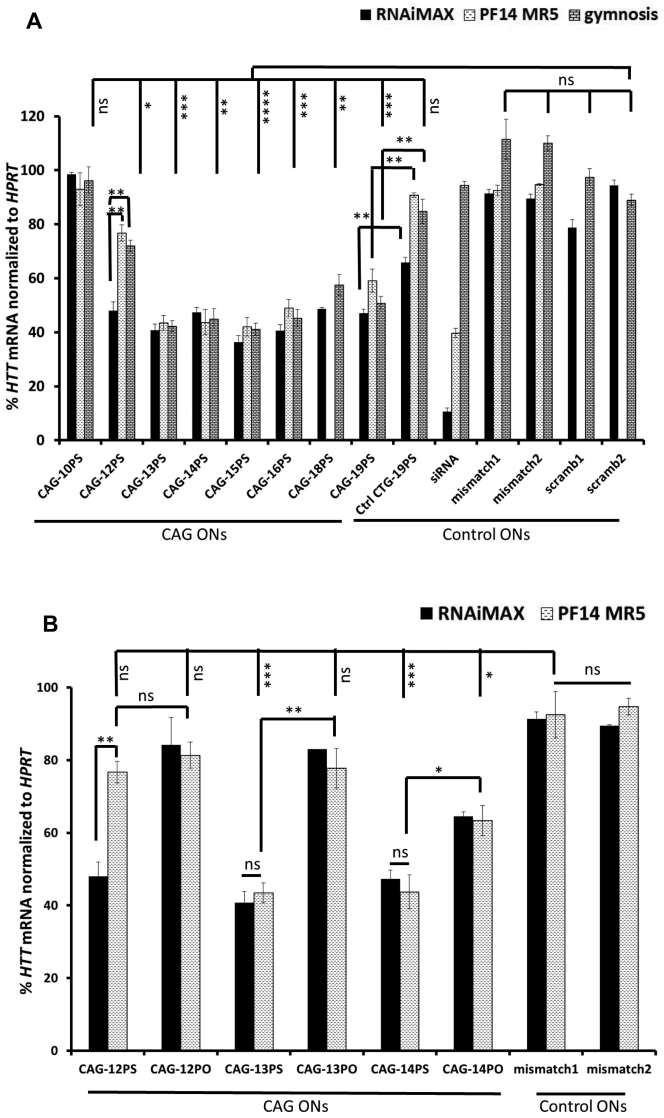

Figure 9. CAG ONs can be delivered to the patient fibroblasts by peptide and gymnotic delivery. (**A**) LNA/DNA CAG 10- to 19-mer PS ONs were transfected at 100 nM into GM04281 human HD fibroblasts using RNAiMAX or PF14 at molar ratio 1:5 (ON: peptide) or were added to the cells at 1 μM without transfection reagents (gymnosis). Control CTG-19PS, mismatched, scrambled ONs and siRNA were used as controls. Four days post treatments, the cells were lysed and RNA was isolated and analyzed by q RT-PCR using primers and probes for *HTT* and *HPRT* as a housekeeping reference gene. Significant differences were found between CAG-19PS and CTG-19PS using all delivery methods (RNAiMAX ($P < 0.01$), PF14 ($P < 0.01$), gymnosis ($P < 0.0001$)). All ONs ranged from 12 to 19-mer delivered by gymnosis significantly reduced HTT mRNA (significance levels are shown in the graph). (**B**) LNA/DNA CAG 12-, 13- or 14-mer ONs with either PS or PO backbone were transfected at 100 nM concentration after formulation with RNAiMAX or with PF14 at molar ratio 1:5 (ON: peptide) into human HD patient fibroblasts GM04281. There was no significant (ns) difference between RNAiMAX and PF14 formulations with CAG-13PS and CAG-14PS. For PF14 formulations, the mismatched controls differed significantly from all treatments except for CAG-12PS, CAG-12PO and CAG-13PO (significance levels for all ONs compared to mismatched 1 are shown in the figure).

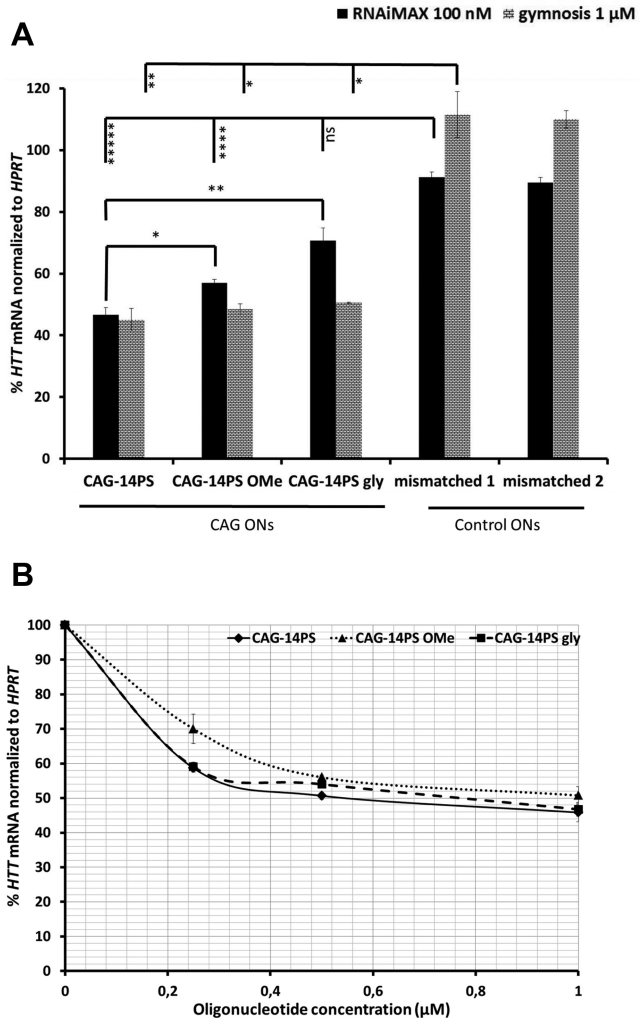

Figure 10. Effect of ON chemical modifications on efficiency. (**A**) CAG-14PS, CAG-14PS-OMe and CAG-14PS-gly were transfected at 100 nM into GM04281 human HD fibroblasts using RNAiMAX, or were added to the cells at 1 μM concentration without transfection reagents (gymnosis). Four days post treatments, the cells were lysed and RNA was isolated and analyzed by q RT-PCR using primers and probes for *HTT* and *HPRT*. Significant differences were found between mRNA levels after treatment with: CAG-14PS versus CAG-14PS-OMe ($P < 0.05$), CAG-14PS versus CAG-14PS-gly ($P < 0.01$). Significance levels of all ONs compared to mismatched 1 ON are shown in the figure. (**B**) A dose response curve for the three ONs after gymnosis with increasing concentrations of each ON in GM04281.

The down-regulation achieved by the CAG ONs, on both HTT mRNA and protein, can be directly expected from their sequence, since they should bind to the complementary DNA strand in the gene. In theory, mRNA, which is a CAG sequence itself, can therefore not be a target for a CAG ON. This was verified by the thermal UV melting measurements, which clearly demonstrated the difference between the CAG and CTG ONs, when incubated with the CAG RNA and, how that affected the shift in *T*m values (Figure 6) as well as by gel shift experiments (data not shown). To further exclude the probability that our CAG ONs could bind the sense RNA sequence, we compared them with CTG ONs by transfection into HD

patient fibroblasts. In fact, CTG ONs were earlier used to down-regulate HTT on the protein level via binding to the *HTT* mRNA and blocking its translation (27). Transfection of CTG-19PS resulted in some mRNA down-regulation, which might be explained by the possible RNase H degradation of mRNA due to the ON sequence that alternates one LNA with two DNA bases (27). This down-regulation was still not efficient; confirming that a gap of at least five DNAs is needed in order to induce a decent RNase H degradation (67). In contrast, the *HTT* mRNA knockdown obtained by CAG-19PS was significantly greater, which strongly suggests that mRNA degradation is not the mechanism by which the CAG ONs affect the mRNA level. Both CAG-19 and CTG-19 showed pronounced protein down-regulation, which was significantly selective for the muHTT protein after 4 and 7 days (Figure 5). The CTG-13PS control ONs did not induce any reduction of the *HTT* mRNA. Unlike the CTG-19PS, none of CTG-13PS ONs caused any reduction of the HTT protein, implying the need for a certain length of the antisense ONs to achieve steric blocking of the mRNA and impair its translation. Conversely, CAG-13PS produced a marked and highly significant down-regulation of both mRNA and protein, indicating the possibility to target DNA with rather short ONs and still obtain an effect. This is advantageous when addressing formulation and delivery aspects of ONs for pharmaceutical applications.

Earlier reports have shown that dsDNA containing CAG repeats, after strand-separation, can adopt a specific secondary structure due to intra-strand, semi-complementary base-pairing (68,69). Since it was unclear whether the examined CAG ONs could bind to these regions, we decided to investigate that further. Steric blocking of restriction enzyme sites has been used previously as a tool to assess ON sequence-specific binding of dsDNA through the formation of a triplex structure (51,52). Here, we show that CAG-ONs could inhibit the AvrII cleavage of a DNA plasmid containing 72 trinucleotide repeats, where the cleavage site of AvrII is located one base-pair outside of the repeat region. The longer the ON, the higher was the inhibition of cleavage at this site. This is expected, since shorter ONs would presumably hybridize too weakly if they would extend outside the target site, and demonstrates that the CAG-ONs could indeed bind to the repeat region in dsDNA. Furthermore, in a study performed by Chung *et al.* (70), a natural antisense transcript consisting of exon 1 and 2 of the *HTT* gene was identified. However, it was shown in the same study that down-regulating this transcript would rather lead to an increase in the endogenous *HTT* transcript levels, suggesting that this transcript is not the target of the investigated CAG ONs.

In order to confirm the DNA binding of the CAG ONs, we investigated the possible occurrence of any epigenetic changes. We first checked the active histone markers: H3K4me3 and H3K27ac, which are associated with TSS of actively, transcribed genes. Level of these markers did not significantly change by the CAG ON treatment, which can be explained by the fact that the CAG ON's effect is independent of promoter activation and silencing. However, a trend towards increased histone acetylation ($P = 0.67$) was still observed at the region immediately upstream of the transcribed trinucleotide repeat sequence in cells transfected with the *HTT* gene targeting ON (Supplementary Figure S5). We hypothesized that this may be linked to slower transcriptional elongation rate at this region of the *HTT* gene leading to slight hyper-acetylation of chromatin upstream of the transcribed repeats.

Since the CAG ONs are targeting exon 1 downstream of the polymerase initiation site, we reasoned that their binding would more likely affect the polymerase elongation. The occupancy of Pol II ser2 is correlated to active transcriptional elongation and is therefore correlated with gene coding regions of actively transcribed genes (71). The significant reduction in ser2 occupancy by CAG-19PS compared to a scrambled ON in both the middle and 3′ end of the *HTT* gene clearly suggests an effect on the DNA level. Altogether, these results provide strong evidence for the anti-gene mechanism as the mode of action of our designed CAG ONs.

Since delivery is a hurdle for most of the ON-based therapeutics, we sought to test methods, other than the commercial cationic lipids, that can be further used for *in vivo* applications of the CAG ONs. PF14 is a recently developed CPP which shows pronounced efficiency as a vehicle for a number of ON categories, such as splice switching ONs (55) and siRNAs (72); however, it was never tested for delivery of anti-gene ONs. Interestingly, similar efficiency was achieved for CAG ONs ranging from 13- to 19-mers as compared to that obtained with RNAiMAX formulations of the same ONs. A number of ONs designed for treatment of HD have been delivered as naked ONs into the brain (26,31). Gymnosis is a technique that has been recently used to simulate the *in vivo* injection of non-formulated ONs but in cell culture models (73). Our LNA/DNA CAG PS ONs showed marked efficiency after gymnosis at a relatively low dose (sub-micromolar range) in human HD fibroblasts. Antisense ONs built of chemically modified nucleotides such as LNA and peptide nucleic acids have previously shown efficiency after gymnosis in cell cultures at similar concentrations (74).

Chemical modification of ONs is commonly used to improve their binding and pharmacokinetic properties, such as the 2′-*O*-Me RNA modification (75,76). Interestingly, we found here that exchanging all DNA bases in the CAG-14PS with 2′-*O*-methyl RNA did not improve the ON down-regulation efficiency. It was previously reported by Wengel *et al.* that the 2′-*O*-methyl RNA interaction with an RNA target is stronger than with a DNA target based on *T*m measurements, which could help to explain the reduced efficiency of the tested ON (77). 2′-Glycylamino-LNA is another recently developed modification carrying a positively charged glycyl group at the N2′-position enhancing duplex stability (78), but did not show any improvement in the anti-gene context. It is plausible that these delivery methods are not optimal for positively charged modified ONs, and further optimization might be needed. It is also possible that such ONs behave differently when administered *in vivo*.

A number of different ON-based strategies have been attempted with the goal of reducing the mu*HTT* expression levels. Targeting mRNA is a very appealing concept because of the wide experience of such methods. In addition, gene targeting can be as well achieved at the transcriptional level using a number of techniques such as zinc finger nucleases, the recently developed CRISPR/Cas systems

(Shin, J.W. *et al*. (2016) Permanent inactivation of Huntington's disease mutation by personalized allele-specific CRISPR/Cas9. *Hum. Mol. Genet*.), or simply by dsDNA strand-invading ONs such as the LNA/DNA mixmers evaluated in this study. Zinc finger nucleases and CRISPR/Cas induce permanent changes in the DNA, but are difficult to deliver. Strand-invading ONs potentially cause a more sustained down-regulation of HTT expression as compared to ONs targeting RNA. Each strategy has its pros and cons and therefore, it can be relevant in the future to consider combined HD therapies formed of ONs working by different mechanisms, since this could reduce toxicity and enhance efficacy.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. A patent application related to these results has been filed with E.M.Z, O.G., P.M.D.M, R.Z., J.W., K.E.L. and C.I.E.S. named as co-inventors.

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